

# Increased Salt-Sensitivity in Endothelial Nitric Oxide Synthase-Knockout Mice

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**Background:** Although impaired nitric oxide production contributes importantly to salt-sensitivity, the role of the endothelial isoform of nitric oxide synthase (eNOS) has received little attention. In the present study we compared the effects of a high-salt diet on the blood pressure response of eNOS knockout (eNOS<sup>-/-</sup>) and control (eNOS<sup>+/+</sup>) mice.

**Methods:** Mean arterial pressure (MAP), heart rate, pulse pressure, and activity levels were recorded by telemetry in mice fed a regular-salt diet (0.7% NaCl) followed by 6 weeks on either a high-salt (8% NaCl) or regular-salt diet.

**Results:** The eNOS<sup>-/-</sup> mice exhibited a 15% increase in MAP and a 2- to 2.5-fold increase in salt-sensitivity relative to the control strain. Salt-induced increases in MAP were well sustained in eNOS<sup>-/-</sup>, whereas in

eNOS<sup>+/+</sup> the initial increase was biphasic. The effects of salt on MAP were particularly pronounced during locomotor activity, during the dark phase, and at the peak levels of MAP recorded over the course of the day. The high-salt diet also led to a transient increase in the proportion of time spent active. Levels of heart rate and pulse pressure were relatively unaffected by the high-salt diet.

**Conclusion:** The eNOS<sup>-/-</sup> mice exhibit an increased blood pressure response to a high-salt diet. This finding suggests that eNOS normally provides an important contribution to the body's adaptation to a salt load and that reduced production of NO by eNOS may promote salt-sensitivity and salt-induced hypertension.

**Key Words:** Sodium-dependent hypertension, mice, nitric oxide, nitric oxide synthase, blood pressure.

Impaired production of nitric oxide (NO) contributes importantly to salt-induced hypertension.<sup>1</sup> Salt-induced increases in NO production are attenuated in animal models of salt-sensitivity.<sup>1-3</sup> In such cases, administration of L-arginine restores NO production and reduces salt-sensitivity.<sup>1,4-6</sup> Conversely, NO synthase (NOS) inhibition produces salt-sensitivity in otherwise salt-resistant animals.<sup>7-10</sup> Similar associations between NO production and salt-sensitivity have been described in hypertensive patients.<sup>11-16</sup>

Nitric oxide is produced by three isoforms of NO synthase. Each isoform has the potential to influence the regulation of blood pressure (BP) through their presence within the vascular endothelium (eNOS [NOS-3]), nervous system (nNOS [NOS-1]), and kidney including the renal tubules (eNOS, iNOS [NOS-2], and nNOS) and macula densa (nNOS).<sup>17</sup> The use of NOS inhibitors with relative specificity for nNOS or iNOS<sup>9,10,18</sup> implicates reduced NO production by these two NOS isoforms as possible contributors to salt-induced hypertension. An impact of eNOS on salt-sensitivity might also be anticipated

based on its influence on the renal cortical and medullary circulations<sup>19</sup> and renal tubular salt transport<sup>20</sup> and its contribution to the actions of several natriuretic substances.<sup>21-23</sup> The objective of the present study was to test the hypothesis that mice with targeted disruption of the eNOS gene would have an increased BP response to salt loading. In contrast to recently published study findings,<sup>24</sup> our results demonstrate a 2- to 2.5-fold enhancement of salt-sensitivity in eNOS knockout mice, suggesting that the eNOS isoform contributes importantly to the BP response to salt loading.

## Methods

### Animals

Control mice (eNOS<sup>+/+</sup>, C57BL/6J) and eNOS-deficient mice<sup>25</sup> (eNOS<sup>-/-</sup>, B6.129P2-NOS<sup>tm1Unc</sup>; backcrossed to a C57BL/6J background) were bred locally or purchased from Jackson Laboratories. Mice were fed a standard regular-salt (RS) feed containing 0.4% sodium (0.7% sodium chloride), 1.0% calcium, 0.9% potassium and 0.8%

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**Table 1.** Control period hemodynamics in the four groups of mice

Characteristic	Control mice		eNOS knockout mice	
	Control diet group (n = 7)	High salt diet group (n = 7)	Control diet group (n = 8)	High salt diet group (n = 6)
24-h MAP (mm Hg)	108 ± 1	105 ± 1	123 ± 2*	121 ± 2*
Light-period MAP (mm Hg)	100 ± 2	97 ± 1	113 ± 2*	113 ± 1*
Dark-period MAP (mm Hg)	116 ± 1	112 ± 1	131 ± 3*	130 ± 3*
Range of 24 h MAP (mm Hg)	54 ± 2	54 ± 2	79 ± 8*	71 ± 5
24-h Minimum MAP (mm Hg)	83 ± 1	81 ± 1	89 ± 1*	90 ± 2
24-h Maximum MAP (mm Hg)	137 ± 1	135 ± 1	168 ± 7*	161 ± 3*
24-h Pulse pressure (mm Hg)	30 ± 1	31 ± 2	33 ± 2	34 ± 2.4
24-h Heart rate (beats/min)	578 ± 10	542 ± 11	547 ± 9	571 ± 10
24-h Active time (% time active)	37 ± 2	36 ± 3	43 ± 5	40 ± 1.5
24-h Intensity of activity (a.u.)	1.2 ± 0.1	1.2 ± 0.0	1.2 ± 0.1	1.2 ± 0.1

a.u. = arbitrary units; MAP = mean arterial pressure.

\*  $P < .05$  between strains, within the same diet group.

phosphorus (Lab diet 5P00, Richmond, IN). A high-salt (HS) diet was created by added additional salt to bring the level to 8% NaCl. The recording room had an average temperature of  $23^{\circ} \pm 0.5^{\circ}\text{C}$  and was maintained on a 12-h light, 12-h dark cycle. Experiments were approved by the Memorial University of Newfoundland Animal Care Committee.

## Experimental Protocol

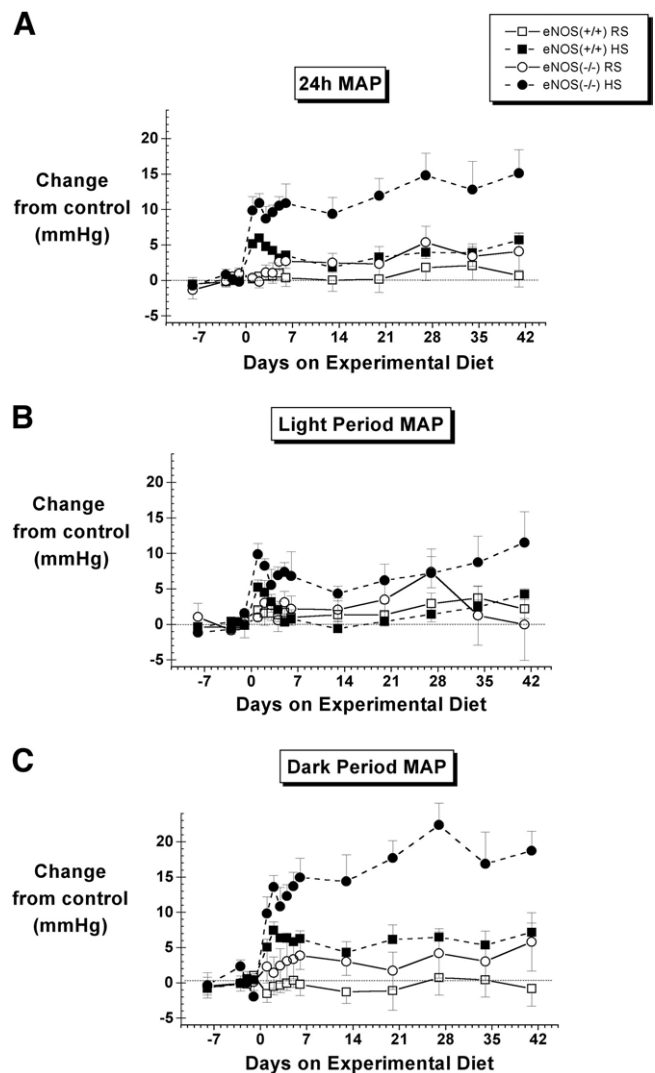
Mice (male, 11 to 18 weeks) were implanted with a telemeter (DSI #TA11PA-C20) via the carotid artery under ketamine/xylazine anesthesia (90 and 10 mg/kg intraperitoneally).<sup>26</sup> The experimental diet was initiated 17 to 21 days after telemeter implantation. The BP data were recorded by telemetry 7 days, 2 days, and 1 day before, and 1 to 6, 13, 20, 27, 34, and 41 days after the switch to the experimental diet.

## Telemetry

Data was sampled at 500 Hz for 3 sec at 30-sec intervals.<sup>26</sup> The 24-h data sets were processed using in-house routines (download link: <http://www.med.mun.ca/basic/pages/faculty/vanvliet.htm>). The average of the offset measured at  $37^{\circ}\text{C}$  before implantation ( $2.9 \pm 0.4$  mm Hg) and after removal ( $3.9 \pm 0.4$  mm Hg) was subtracted from the collected data before analysis. The proportion of time spent active (Active Time) was calculated by dividing the number of sample periods for which the activity signal was  $>0$  by the total number of samples. The intensity of activity was calculated<sup>26</sup> as the mean of log (activity) for values of activity  $>0$ .

## Statistical Analysis

Data are reported as the mean  $\pm$  SEM. The protocol was divided into four phases for statistical analysis: 1) control, 2) early (week 1), 3) middle (weeks 2–3), and 4) late (4–6). The Dunnett test was used for comparisons between control and experimental periods. A general linear model analysis of variance followed by a Tukey test was



**FIG. 1.** The effect of regular-salt (RS) versus high-salt (HS) diet on mean arterial pressure (MAP) in control (eNOS+/+) and eNOS knockout (eNOS−/−) mice. Data are shown for the average of the entire day (24-h MAP, **top**) as well as the individual 12-h light and 12-h dark periods of the day (**middle** and **bottom**, respectively).  $N = 6$  to 8 except on day 2 for which  $N = 4$  for the eNOS−/− HS group. Control values are given in Table 1. See Table 2 for statistical comparisons.

**Table 2.** Between-group comparisons of salt-induced responses

Characteristic	Early phase (week 1)	Middle phase (weeks 2-3)	Late phase (weeks 4-6)
24-h MAP			
eNOS <sup>-/-</sup> RS v eNOS <sup>-/-</sup> HS	*	*	*
eNOS <sup>-/-</sup> HS v eNOS <sup>+/+</sup> HS	*	*	*
eNOS <sup>-/-</sup> RS v eNOS <sup>+/+</sup> RS	NS	NS	NS
eNOS <sup>+/+</sup> RS v eNOS <sup>+/+</sup> HS	*	NS	NS
Light-period MAP			
eNOS <sup>-/-</sup> RS v eNOS <sup>-/-</sup> HS	*	NS	*
eNOS <sup>-/-</sup> HS v eNOS <sup>+/+</sup> HS	*	NS	*
eNOS <sup>-/-</sup> RS v eNOS <sup>+/+</sup> RS	NS	NS	NS
eNOS <sup>+/+</sup> RS v eNOS <sup>+/+</sup> HS	NS	NS	NS
Dark-period MAP			
eNOS <sup>-/-</sup> RS v eNOS <sup>-/-</sup> HS	*	*	*
eNOS <sup>-/-</sup> HS v eNOS <sup>+/+</sup> HS	*	*	*
eNOS <sup>-/-</sup> RS v eNOS <sup>+/+</sup> RS	NS	NS	NS
eNOS <sup>+/+</sup> RS v eNOS <sup>+/+</sup> HS	*	NS	*
Range of MAP			
eNOS <sup>-/-</sup> RS v eNOS <sup>-/-</sup> HS	NS	*	*
eNOS <sup>-/-</sup> HS v eNOS <sup>+/+</sup> HS	NS	NS	NS
eNOS <sup>-/-</sup> RS v eNOS <sup>+/+</sup> RS	NS	NS	NS
eNOS <sup>+/+</sup> RS v eNOS <sup>+/+</sup> HS	*	NS	NS
Minimum MAP			
eNOS <sup>-/-</sup> RS v eNOS <sup>-/-</sup> HS	NS	NS	NS
eNOS <sup>-/-</sup> HS v eNOS <sup>+/+</sup> HS	*	*	*
eNOS <sup>-/-</sup> RS v eNOS <sup>+/+</sup> RS	NS	NS	*
eNOS <sup>+/+</sup> RS v eNOS <sup>+/+</sup> HS	NS	NS	NS
Maximum MAP			
eNOS <sup>-/-</sup> RS v eNOS <sup>-/-</sup> HS	*	*	*
eNOS <sup>-/-</sup> HS v eNOS <sup>+/+</sup> HS	*	NS	*
eNOS <sup>-/-</sup> RS v eNOS <sup>+/+</sup> RS	NS	NS	NS
eNOS <sup>+/+</sup> RS v eNOS <sup>+/+</sup> HS	*	NS	NS
24-h Pulse pressure			
eNOS <sup>-/-</sup> RS v eNOS <sup>-/-</sup> HS	NS	NS	NS
eNOS <sup>-/-</sup> HS v eNOS <sup>+/+</sup> HS	NS	NS	NS
eNOS <sup>-/-</sup> RS v eNOS <sup>+/+</sup> RS	NS	NS	NS
eNOS <sup>+/+</sup> RS v eNOS <sup>+/+</sup> HS	NS	NS	*
24-h heart rate			
eNOS <sup>-/-</sup> RS v eNOS <sup>-/-</sup> HS	NS	NS	*
eNOS <sup>-/-</sup> HS v eNOS <sup>+/+</sup> HS	NS	NS	*
eNOS <sup>-/-</sup> RS v eNOS <sup>+/+</sup> RS	NS	*	*
eNOS <sup>+/+</sup> RS v eNOS <sup>+/+</sup> HS	NS	NS	NS
Active time			
eNOS <sup>-/-</sup> RS v eNOS <sup>-/-</sup> HS	*	NS	NS
eNOS <sup>-/-</sup> HS v eNOS <sup>+/+</sup> HS	NS	NS	NS
eNOS <sup>-/-</sup> RS v eNOS <sup>+/+</sup> RS	NS	NS	NS
eNOS <sup>+/+</sup> RS v eNOS <sup>+/+</sup> HS	*	NS	NS

HS = high salt diet; NS = not significant; RS = regular salt diet. Intensity of Activity results are not shown (ANOVA, NS).

\*  $P < .05$  for specified comparison (Tukey test).

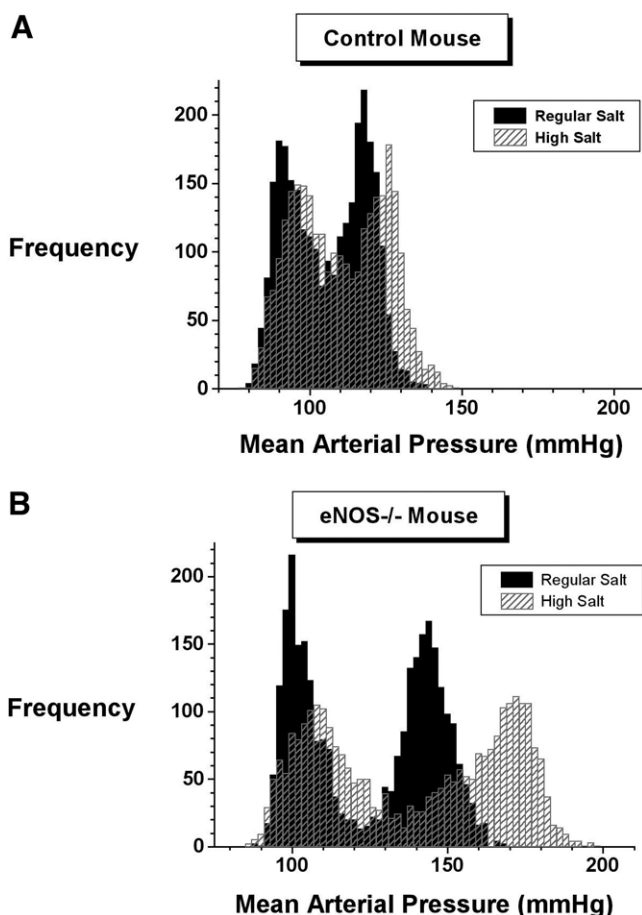
used to evaluate differences between groups. A value of  $P < .05$  was used as the limit of statistical significance.

## Results

### Changes in MAP Values

The eNOS<sup>-/-</sup> mice were hypertensive, with significant increases in their 24-h MAP, 12-h light-period MAP, 12-h dark period MAP, and daily maximum MAP (Table 1). MAP was significantly increased by the high-salt diet in both eNOS<sup>+/+</sup> and eNOS<sup>-/-</sup> mice (Fig. 1, Table 2). In

eNOS<sup>+/+</sup> mice, the increase in MAP was relatively modest ( $5.9 \pm 0.6$  mm Hg at day 2) and biphasic, the response being significant during the early phase (24-h and dark-period data) and late phases (dark-period data). In contrast, the response of eNOS<sup>-/-</sup> mice was significantly greater and well sustained. After initially peaking on day 2 ( $10.8 \pm 1.3$  mm Hg above control levels), the 24-h MAP slowly climbed to the overall peak response occurring on the last day of the experiment ( $15.1 \pm 3.3$  mm Hg above control levels). The effects of salt on MAP were most pronounced during the dark period of the day (Fig. 1, Table 1).



**FIG. 2.** Frequency distributions of mean arterial pressure in a typical control (eNOS+/+, **top**) and eNOS knockout mouse (eNOS-/-, **bottom**). Distributions are shown for the control period (regular-salt diet, **dark columns**) and on the final day (day 42) of the high-salt diet (**hatched columns**).

### Distribution of MAP Values

The BP of C57Bl/6J mice was bimodally distributed (Fig. 2), the left and right modes of the distribution corresponding to values recorded during periods of inactivity and activity, respectively.<sup>26</sup> After 6 weeks on the high-salt diet, the BP distribution of eNOS-/- mice was wider, with significant increases in the daily maximum MAP and daily MAP range, but little to no change in the daily minimum MAP level (Fig. 3, Table 2). In contrast, little to no change was observed in the MAP distributions of eNOS+/+ mice fed a high-salt diet (Figs. 2 and 3, Table 2).

### Pulse Pressure

Pulse pressure tended to increase on the high-salt diet, but this tendency reached significance only in the case of the eNOS+/+ mice (Fig. 4, Table 2).

### Heart Rate

In eNOS-/- mice, the high-salt diet was associated with a tendency for the HR to decrease from that of the control group, reaching significance in the late phase (Fig. 4,

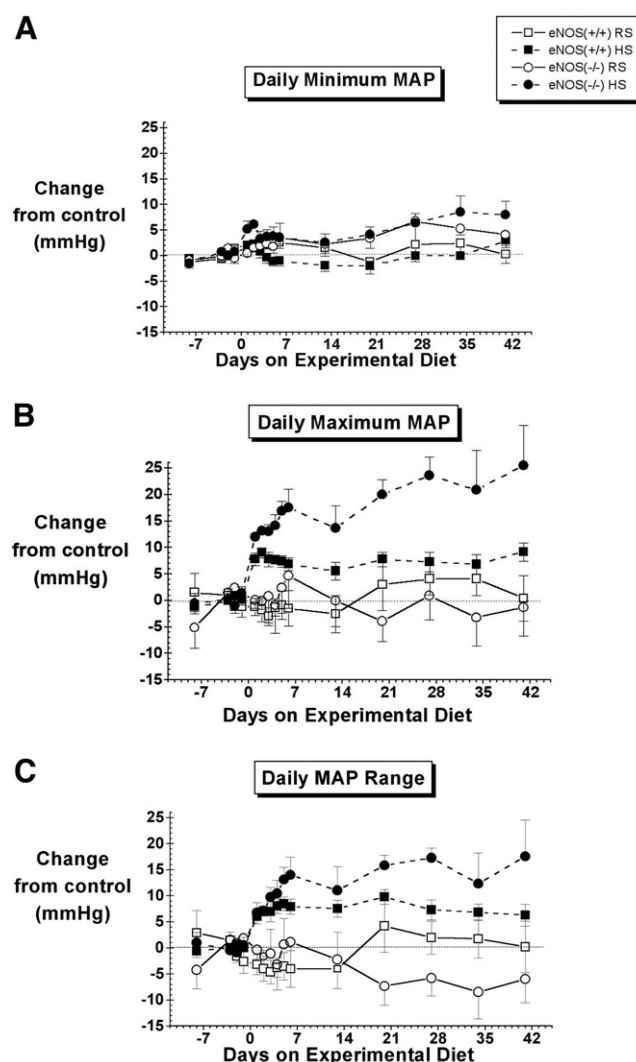
Table 2). No significant changes were observed in the heart rate of eNOS+/+ mice.

### Locomotor Activity

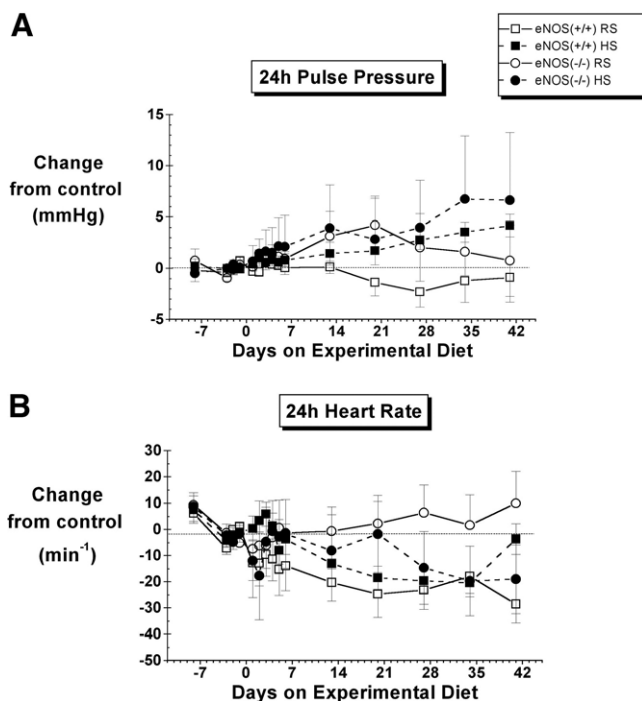
In both the eNOS+/+ and eNOS-/- mice, the percentage of time spent active initially increased when the mice were placed on the high-salt diet and gradually decreased toward baseline levels as the protocol progressed (Fig. 5, Table 2). No significant changes were observed in the intensity of activity (Fig. 5, Table 2).

### Discussion

Impaired nitric oxide production is associated with salt-sensitivity of BP.<sup>1</sup> However, the role of individual NOS isoforms is not well understood. Our data demonstrate that



**FIG. 3.** Effect of a regular-salt (RS) versus high-salt (HS) diet on the daily range, minimum and maximum mean arterial pressure (MAP) in control (eNOS+/+) and eNOS knockout (eNOS-/-) mice. *N* = 6 to 8 except on day 2 for which *N* = 4 for the eNOS-/- HS group. Control values are given in Table 1. Statistical comparisons are shown in Table 2.



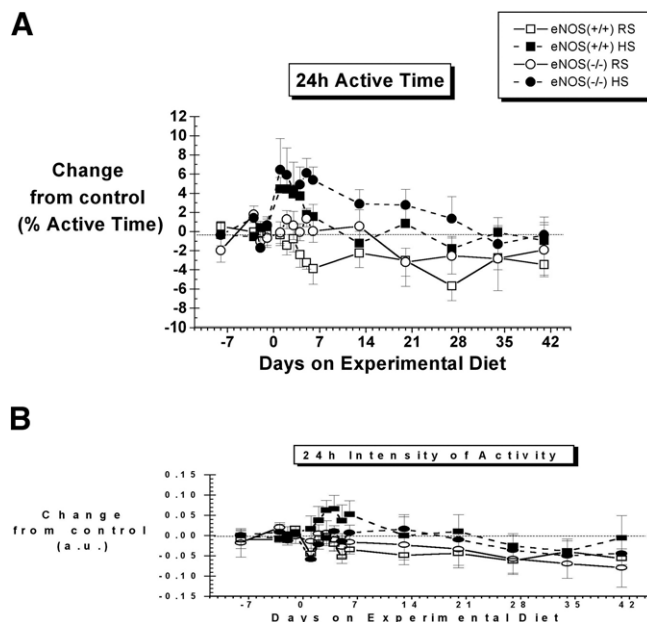
**FIG. 4.** The effect of a regular-salt (RS) and high-salt (HS) diet on 24-h pulse pressure and heart rate in control (eNOS+/+) and eNOS knockout (eNOS-/-) mice.  $N = 6$  to 8 except on day 2 for which  $N = 4$  for the eNOS-/- HS group. Control values are given in Table 1. See Table 2 for statistical comparisons.

eNOS knockout mice exhibit increased salt-sensitivity of BP. This finding suggests that, in addition to its roles as a regulator of regional blood flow and basal BP, the eNOS

isoform also participates in the body's adaptation to a salt load. In turn, this suggests that impaired production of NO by eNOS could contribute importantly to salt sensitivity and salt-induced hypertension.

Salt-induced increases in the BP of eNOS-/- mice were  $\sim 2$  to 2.5 fold greater than that of the control strain (Fig. 1). This effect of salt appeared to be caused by a pronounced rightward expansion of the MAP distribution with little to no change in the left-hand edge of the distribution (Fig. 2). This conclusion is consistent with the observed increases in the maximum and 24-h mean MAP level, and the absence of any significant change in the minimal MAP level. Consistent with previous reports in rats<sup>27,28</sup> and mice,<sup>29</sup> the greatest effects of salt on MAP occurred during the dark phase of the day (Fig. 1) and during bouts of locomotor activity, irrespective of the time of day (data not shown).

Our conclusion differs markedly from that of a recent study by Mattson and Meister.<sup>24</sup> In their study, eNOS-/- mice of the same sex, genetic background, and origin were reported to be completely salt resistant. The two studies differed in many respects. In the study by Mattson and Meister,<sup>24</sup> BP was measured by catheter during brief (3-h/day) recordings made over a 3-day control and 3-day experimental period, 3 to 5 days after catheter installation. In contrast, the present study was based on continuous recordings by telemetry from undisturbed mice commencing 10 to 14 days after telemeter implantation and continuing during 42 days exposure to the high-salt diet. Heart rates differed markedly between the two studies ( $640$  to  $667$  min<sup>-1</sup> v  $<580$  min<sup>-1</sup> in the present study). However,



**FIG. 5.** The effect of a regular-salt (RS) versus high-salt (HS) diet on the percentage and level (intensity) of activity in eNOS+/+ and eNOS-/- mice.  $N = 6$  to 8 except on day 2 for which  $N = 4$  for the eNOS-/- HS group. Control values are given in Table 1. See Table 2 for statistical comparisons. a.u. = arbitrary units.



the most important difference may be the level of salt loading. In the present study, salt loading was achieved by providing an 8% NaCl diet as is often used in rodent studies. Based on measured food consumption rates in our mice, we estimate the salt load in our experiments to be ~5 mEq Na/day. In contrast, the salt load in the study by Mattson and Meister<sup>24</sup> was ~1 mEq Na/day delivered by intravenous infusion of saline. It is quite possible that we would not have observed salt-sensitivity if we had used this lower level of salt loading. However, it is important to note that the 8% salt diet used in our study was sufficient to elevate BP even in the control strain of C57Bl/6J mice (consistent with previous studies in this strain<sup>29,30</sup>), whereas control mice showed no response to salt loading in the study by Mattson and Meister.<sup>24</sup> This is an essential point, as salt resistance in the control mice has the potential to mask an effect of a gene on salt-sensitivity. The moderate salt-sensitivity displayed by our control mice while consuming an 8% salt diet suggests that this protocol is highly appropriate for investigating the genetic determinants of salt-sensitivity in mice of a C57Bl/6J background.

In summary, our results suggest that the eNOS isoform provides an important contribution to the BP response to salt-loading. Reductions in NO production by eNOS (eg, because of quenching by reactive oxygen species, endogenous NOS inhibitors, substrate or cofactor limitations) may promote salt-sensitivity and salt-induced hypertension.

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